

METHODS FOR THE IDENTIFICATION OF INHIBITORS OF  
ORNITHINE CARBAMOYLTRANSFERASE AS ANTIBIOTICS

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RELATED APPLICATION

This application claims the benefit of U.S. Provisional Application No. 60/472,242, filed May 21, 2003 and incorporated in its entirety by reference.

FIELD OF THE INVENTION

10 The invention relates generally to methods for the identification of antibiotics, preferably antifungals that affect the biosynthesis of arginine.

BACKGROUND OF THE INVENTION

Filamentous fungi are causal agents responsible for many serious pathogenic infections of plants and animals. Since fungi are eukaryotes, and thus more similar to their host organisms than, for example bacteria, the treatment of infections by fungi poses special risks and challenges not encountered with other types of infections. One such fungus is *Magnaporthe grisea*, the fungus that causes rice blast disease, a significant threat to food supplies worldwide. Other examples of plant pathogens of economic importance include the pathogens in the genera *Agaricus*, *Alternaria*, *Anisogramma*, *Anthracoidea*, *Antrodia*, *Apiognomonia*, *Apiosporina*, *Armillaria*, *Ascochyta*, *Aspergillus*, *Bipolaris*, *Bjerkandera*, *Botryosphaeria*, *Botrytis*, *Ceratobasidium*, *Ceratocystis*, *Cercospora*, *Cercosporidium*, *Cerotelium*, *Cerrena*, *Chondrostereum*, *Chryphonectria*, *Chrysomyxa*, *Cladosporium*, *Claviceps*, *Cochliobolus*, *Coleosporium*, *Colletotrichium*,

*Colletotrichum, Corticium, Corynespora, Cronartium, Cryphonectria, Cryptosphaeria, Cyathus, Cymadothea, Cytospora, Daedaleopsis, Diaporthe, Didymella, Diplocarpon, Diplodia, Discohainesia, Discula, Dothistroma, Drechslera, Echinodontium, Elsinoe, Endocronartium, Endothia, Entyloma, Epichloe, Erysiphe, Exobasidium, Exserohilum, Fomes, Fomitopsis, Fusarium, Gaeumannomyces, Ganoderma, Gibberella, Gloeocercospora, Gloeophyllum, Gloeoporus, Glomerella, Gnomoniella, Guignardia, Gymnosporangium, Helminthosporium, Herpotrichia, Heterobasidion, Hirschioporus, Hypodermella, Inonotus, Irpex, Kabatiella, Kabatina, Laetiporus, Laetisaria, Lasiodiplodia, Laxitextum, Leptographium, Leptosphaeria, Leptosphaerulina, Leucytespora, Linospora, Lophodermella, Lophodermium, Macrohomina, Magnaporthe, Marssonina, Melampsora, Melampsorella, Meria, Microdochium, Microsphaera, Monilinia, Monochaetia, Morchella, Mycosphaerella, Myrothecium, Nectria, Nigrospora, Ophiophaerella, Ophiostoma, Penicillium, Perenniporia, Peridermium, Pestalotia, Phaeocryptopus, Phaeolus, Phakopsora, Phellinus, Phialophora, Phoma, Phomopsis, Phragmidium, Phyllachora, Phyllactinia, Phyllosticta, Phymatotrichopsis, Pleospora, Podosphaera, Pseudopeziza, Pseudoseptoria, Puccinia, Pucciniastrum, Pyricularia, Rhabdocline, Rhizoctonia, Rhizopus, Rhizosphaera, Rhynchosporium, Rhytisma, Schizophyllum, Schizopora, Scirrhia, Sclerotinia, Sclerotium, Scytinostroma, Septoria, Setosphaera, Sirococcus, Spaerotheca, Sphaeropsis, Sphaerotheca, Sporisorium, Stagonospora, Stemphylium, Stenocarpella, Stereum, Taphrina, Thielaviopsis, Tilletia, Trametes, Tranzschelia, Trichoderma, Tubakia, Typhula, Uncinula, Urocystis, Uromyces, Ustilago, Valsa, Venturia, Verticillium, Xylaria, and others. Related organisms are classified in the oomycetes classification and include the genera *Albugo*, *Aphanomyces*, *Bremia*, *Peronospora*, *Phytophthora*, *Plasmodiophora*, *Plasmopara*, *Pseudoperonospora*, *Pythium*, *Sclerotiora*, and others. Oomycetes are also significant plant pathogens and are sometimes classified along with the true fungi.*

Human diseases that are caused by filamentous fungi include life-threatening lung and disseminated diseases, often a result of infections by *Aspergillus fumigatus*. Other fungal diseases in animals are caused by fungi in the genera *Fusarium*, *Blastomycetes*, *Microsporum*, *Trichophyton*, *Epidermophyton*, *Candida*, *Histoplamsa*, *Pneumocystis*,

*Cryptococcus*, other *Aspergilli*, and others. Control of fungal diseases in plants and animals is usually mediated by chemicals that inhibit growth, proliferation, and/or pathogenicity of fungal organisms. To date, there are less than twenty known modes-of-action for plant protection fungicides and human antifungal compounds.

5 A pathogenic organism has been defined as an organism that causes, or is capable of causing disease. Pathogenic organisms propagate on or in tissues and may obtain nutrients and other essential materials from their hosts. A substantial amount of work concerning filamentous fungal pathogens has been performed with the human pathogen, *Aspergillus fumigatus*. Shibuya *et al.*, 27 Microb. Pathog. 123 (1999) (PubMed Identifier 10 (PMID): 10455003) have shown that the deletion of either of two suspected pathogenicity related genes encoding an alkaline protease or a hydrophobin (rodlet), respectively, did not reduce mortality of mice infected with these mutant strains. Smith *et al.*, 62 Infect. Immun. 5247 (1994) (PMID: 7960101) showed similar results with alkaline protease and the ribotoxin restrictocin; *Aspergillus fumigatus* strains mutated for either of these genes 15 were fully pathogenic to mice. Reichard *et al.*, 35 J. Med. Vet. Mycol. 189 (1997) (PMID: 9229335) showed that deletion of the suspected pathogenicity gene encoding aspergillopepsin (PEP) in *Aspergillus fumigatus* had no effect on mortality in a guinea pig model system, whereas Aufauvre-Brown *et al.*, 21 Fungal. Genet. Biol. 141 (1997) (PMID: 9073488) showed no effects of a chitin synthase mutation on pathogenicity.

20 However, not all experiments produced negative results. Ergosterol is an important membrane component found in fungal organisms. Pathogenic fungi lacking key enzymes in the ergosterol biochemical pathway might be expected to be non-pathogenic since neither the plant nor animal hosts contain this particular sterol. Many antifungal compounds that affect the ergosterol biochemical pathway have been 25 previously described. (United States Patent Nos. 4,920,109; 4,920,111; 4,920,112; 4,920,113; and 4,921,844; Hewitt, H. G. Fungicides in Crop Protection Cambridge, University Press(1998)). D'Enfert *et al.*, 64 Infect. Immun. 4401 (1996) (PMID: 8926121)) showed that an *Aspergillus fumigatus* strain mutated in an orotidine 5'-phosphate decarboxylase gene was entirely non-pathogenic in mice, and Brown *et al.* 30 (Brown *et al.*, 36 Mol. Microbiol. 1371 (2000) (PMID: 10931287)) observed a non-pathogenic result when genes involved in the synthesis of para-aminobenzoic acid were

5 mutated. Some specific target genes have been described as having utility for the screening of inhibitors of plant pathogenic fungi. U.S. Patent No. 6,074,830 to Bacot *et al.*, describe the use of 3,4-dihydroxy-2-butanone 4-phosphate synthase, and U.S. Patent No. 5,976,848 to Davis *et al.* describes the use of dihydroorotate dehydrogenase for potential screening purposes.

There are also a number of papers that report less clear results, showing neither full pathogenicity nor non-pathogenicity of mutants. For example, Hensel *et al.* (Hensel, M. *et al.*, 258 Mol. Gen. Genet. 553 (1998) (PMID: 9669338)) showed only moderate effects of the deletion of the *areA* transcriptional activator on the pathogenicity of 10 *Aspergillus fumigatus*. Therefore, it is not currently possible to determine which specific growth materials may be readily obtained by a pathogen from its host, and which materials may not.

The present invention discloses ornithine carbamoyltransferase as a target for the identification of antifungal, biocide, and biostatic materials. A gene encoding ornithine 15 carbamyltransferase has been cloned from several fungi, including *Aspergillus nidulans* (Upshall, A. *et al.* 204 Mol. Gen. Genet. 349 (1986)); *Aspergillus niger* (Buxton, F.P. *et al.* 60 Gene 255 (1987)); *Neurospora crassa* (Flint, H.J. and Wilkening, J. 203 Mol Gen Genet 110 (1986)); *Saccharomyces cerevisiae* (Huygen, R. *et al.* 166 Eur. J. Biochem. 371 1987)); *Schizosaccharomyces pombe* (Van Huffel, C. *et al.* 205 Eur. J. Biochem. 33 20 (1992)); and *Magnaporthe grisea* (Hamer, L. *et al.* 33 Fungal Genet. Biol. 137 (2001)).

The *A. nidulans* ornithine carbamoyltransferase gene (*ARGB*) has been used as a selection marker in fungal transformations (Johnstone, I.L. *et al.* 4 EMBO J 1307 (1985); Buxton, F.P. *et al.* 37 Gene 207 (1985); Ochsner, U.A. *et al.* 7 Yeast 513 (1991)). In 25 *Fusarium oxysporum* f. sp. *melonis*, a mutation in argininosuccinate synthetase gene *ARG1*, involved in arginine biosynthesis, causes reduced pathogenicity (Namiki, F. *et al.* 14 Mol Plant Microbe. Interact. 580 (2001)).

## SUMMARY OF THE INVENTION

The present inventors have discovered that *in vivo* disruption of the gene encoding 30 ornithine carbamoyltransferase in *Magnaporthe grisea* eliminates the pathogenicity of the fungus. Thus, the present inventors have discovered that ornithine carbamoyltransferase

is useful as a target for the identification of antibiotics, preferably fungicides. Accordingly, the present invention provides methods for the identification of compounds that inhibit ornithine carbamoyltransferase expression or activity. Methods of the invention are useful for the identification of antibiotics, preferably fungicides.

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#### BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Diagram of the reversible reaction catalyzed by ornithine carbamoyltransferase (OCTase). The enzyme catalyzes the reversible interconversion of carbamoyl phosphate and L-ornithine to L-citrulline and phosphate. This reaction is part of the arginine biosynthesis pathway.

Figure 2. Digital image showing the effect of ARG3 gene disruption on *Magnaporthe grisea* pathogenicity using whole plant infection assays. Rice variety CO39 was inoculated with wild-type strain Guy11 and transposon insertion strains, KO1-15 and KO1-23. Leaf segments were imaged at five days post-inoculation.

Figures 3A and 3B. Graphical representation of the growth of wild-type and OCTase mutant *M. grisea* strains in the presence and absence of arginine to verify the loss of ARG3 gene function. Wild-type (Guy11) and transposon insertion strains, KO1-15 and KO1-23, were grown in minimal media (Figure 3A) and minimal media with the addition of arginine (Figure 3B). The x-axis shows time in days and the y-axis shows turbidity measured at 600 nanometers.

#### DETAILED DESCRIPTION OF THE INVENTION

Unless otherwise indicated, the following terms are intended to have the following meanings in interpreting the present invention.

The term "antibiotic" refers to any substance or compound that when contacted with a living cell, organism, virus, or other entity capable of replication, results in a reduction of growth, viability, or pathogenicity of that entity.

The term "antipathogenic," as used herein, refers to a mutant form of a gene that inactivates a pathogenic activity of an organism on its host organism or substantially reduces the level of pathogenic activity, wherein "substantially" means a reduction at least as great as the standard deviation for a measurement, preferably a reduction to 50%

activity, more preferably a reduction of at least one magnitude, i.e. to 10% activity. The pathogenic activity affected may be an aspect of pathogenic activity governed by the normal form of the gene, or the pathway the normal form of the gene functions on, or the pathogenic activity of the organism in general. “Antipathogenic” may also refer to a cell, 5 cells, tissue, or organism that contains the mutant form of a gene; a phenotype associated with the mutant form of a gene, and/or associated with a cell, cells, tissue, or organism that contain the mutant form of a gene.

The term “binding” refers to a non-covalent or a covalent interaction, preferably non-covalent, that holds two molecules together. For example, two such molecules could 10 be an enzyme and an inhibitor of that enzyme. Non-covalent interactions include hydrogen bonding, ionic interactions among charged groups, van der Waals interactions, and hydrophobic interactions among nonpolar groups. One or more of these interactions can mediate the binding of two molecules to each other.

The term "biochemical pathway" or “pathway” refers to a connected series of 15 biochemical reactions normally occurring in a cell, or more broadly a cellular event such as cellular division or DNA replication. Typically, the steps in such a biochemical pathway act in a coordinated fashion to produce a specific product or products or to produce some other particular biochemical action. Such a biochemical pathway requires the expression product of a gene if the absence of that expression product either directly 20 or indirectly prevents the completion of one or more steps in that pathway, thereby preventing or significantly reducing the production of one or more normal products or effects of that pathway. Thus, an agent specifically inhibits such a biochemical pathway requiring the expression product of a particular gene if the presence of the agent stops or substantially reduces the completion of the series of steps in that pathway. Such an agent 25 may, but does not necessarily, act directly on the expression product of that particular gene.

As used herein, the term “conditional lethal” refers to a mutation permitting growth and/or survival only under special growth or environmental conditions.

As used herein, the term “cosmid” refers to a hybrid vector used in gene cloning 30 that includes a cos site (from the lambda bacteriophage). In some cases, the cosmids of

the invention comprise drug resistance marker genes and other plasmid genes. Cosmids are especially suitable for cloning large genes or multigene fragments.

“Fungi” (singular: fungus) refers to whole fungi, fungal organs and tissues (*e.g.*, asci, hyphae, pseudohyphae, rhizoid, sclerotia, sterigmata, spores, sporodochia, 5 sporangia, synnemata, conidia, ascostroma, cleistothecia, mycelia, perithecia, basidia and the like), spores, fungal cells and the progeny thereof. Fungi are a group of organisms (about 50,000 known species), including, but not limited to, mushrooms, mildews, moulds, yeasts, *etc.*, comprising the kingdom Fungi. Fungi exist as single cells or a multicellular body called a mycelium, which consists of filaments known as hyphae.

10 Most fungal cells are multinucleate and have cell walls composed chiefly of chitin. Fungi exist primarily in damp situations on land, and lacking the ability to manufacture their own food by photosynthesis due to the absence of chlorophyll, are either parasites on other organisms or saprotrophs feeding on dead organic matter. Principal criteria used in classification are the nature of the spores produced and the presence or absence of

15 cross walls within the hyphae. Fungi are distributed worldwide in terrestrial, freshwater, and marine habitats. Some fungi live in the soil. Many pathogenic fungi cause disease in animals and man or in plants, while some saprotrophs are destructive to timber, textiles, and other materials. Some fungi form associations with other organisms, most notably with algae to form lichens.

20 As used herein, the term "fungicide," "antifungal," or "antimycotic" refers to an antibiotic substance or compound that kills or suppresses the growth, viability, or pathogenicity of at least one fungus, fungal cell, fungal tissue or spore.

In the context of this disclosure, "gene" should be understood to refer to a unit of heredity. Each gene is composed of a linear chain of deoxyribonucleotides that can be 25 referred to by the sequence of nucleotides forming the chain. Thus, "sequence" is used to indicate both the ordered listing of the nucleotides which form the chain, and the chain having that sequence of nucleotides. "Sequence" is used in the similar way in referring to RNA chains, linear chains made of ribonucleotides. The gene may include regulatory and control sequences, sequences which can be transcribed into an RNA molecule, and 30 may contain sequences with unknown function. The majority of the RNA transcription products are messenger RNAs (mRNAs), which include sequences which are translated

into polypeptides and may include sequences which are not translated. It should be recognized that small differences in nucleotide sequence for the same gene can exist between different fungal strains, or even within a particular fungal strain, without altering the identity of the gene.

5 As used in this disclosure, the terms "growth" or "cell growth" of an organism refer to an increase in mass, density, or number of cells of the organism. Common methods for the measurement of growth include the determination of the optical density of a cell suspension, the counting of the number of cells in a fixed volume, the counting of the number of cells by measurement of cell division, the measurement of cellular mass  
10 or cellular volume, and the like.

As used in this disclosure, the term "growth conditional phenotype" indicates that a fungal strain having such a phenotype exhibits a significantly greater difference in growth rates in response to a change in one or more of the culture parameters than an otherwise similar strain not having a growth conditional phenotype. Typically, a growth  
15 conditional phenotype is described with respect to a single growth culture parameter, such as temperature. Thus, a temperature (or heat-sensitive) mutant (*i.e.*, a fungal strain having a heat-sensitive phenotype) exhibits significantly different growth, and preferably no growth, under non-permissive temperature conditions as compared to growth under permissive conditions. In addition, such mutants preferably also show intermediate  
20 growth rates at intermediate, or semi-permissive, temperatures. Similar responses also result from the appropriate growth changes for other types of growth conditional phenotypes.

As used herein, the term "heterologous OCTase" means either a nucleic acid encoding a polypeptide or a polypeptide, wherein the polypeptide has at least 40%, 45%,  
25 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity or each integer unit of sequence identity from 40-99% in ascending order to *M. grisea* OCTase protein (SEQ ID NO:3) and at least 10%, 25%, 50%, 75%, 80%, 90%, 95%, or 99% activity or each integer unit of activity from 10-100% in ascending order of the activity of the *M. grisea* protein (SEQ ID NO:3).  
30 Examples of heterologous OCTases include, but are not limited to, OCTase from *Neurospora crassa* and OCTase from *Aspergillus terreus*.

As used herein, the term "His-Tag" refers to an encoded polypeptide consisting of multiple consecutive histidine amino acids.

As used herein, the terms "hph," "hygromycin B phosphotransferase," and "hygromycin resistance gene" refer to a hygromycin phosphotransferase gene or gene product.

As used herein, the term "imperfect state" refers to a classification of a fungal organism having no demonstrable sexual life stage.

The term "inhibitor," as used herein, refers to a chemical substance that inactivates the enzymatic activity of OCTase or substantially reduces the level of enzymatic activity, wherein "substantially" means a reduction at least as great as the standard deviation for a measurement, preferably a reduction to 50% activity, more preferably a reduction of at least one magnitude, i.e. to 10% activity. The inhibitor may function by interacting directly with the enzyme, a cofactor of the enzyme, the substrate of the enzyme, or any combination thereof.

A polynucleotide may be "introduced" into a fungal cell by any means known to those of skill in the art, including transfection, transformation or transduction, transposable element, electroporation, particle bombardment, infection, and the like. The introduced polynucleotide may be maintained in the cell stably if it is incorporated into a non-chromosomal autonomous replicon or integrated into the fungal chromosome.

Alternatively, the introduced polynucleotide may be present on an extra-chromosomal non-replicating vector and be transiently expressed or transiently active.

As used herein, the term "knockout" or "gene disruption" refers to the creation of organisms carrying a null mutation (a mutation in which there is no active gene product), a partial null mutation or mutations, or an alteration or alterations in gene regulation by interrupting a DNA sequence through insertion of a foreign piece of DNA. Usually the foreign DNA encodes a selectable marker.

As used herein, the term "mutant form" of a gene refers to a gene which has been altered, either naturally or artificially, by changing the base sequence of the gene. The change in the base sequence may be of several different types, including changes of one or more bases for different bases, deletions, and/or insertions, such as by a transposon. In contrast, a normal form of a gene (wild-type) is a form commonly found in natural

populations of an organism. Commonly a single form of a gene will predominate in natural populations. In general, such a gene is suitable as a normal form of a gene, however, other forms which provide similar functional characteristics may also be used as a normal gene. In particular, a normal form of a gene does not confer a growth conditional phenotype on the strain having that gene, while a mutant form of a gene suitable for use in these methods does provide such a growth conditional phenotype.

5 As used herein, the term “Ni-NTA” refers to nickel sepharose.

As used herein, a “normal” form of a gene (wild-type) is a form commonly found in natural populations of an organism. Commonly a single form of a gene will 10 predominate in natural populations. In general, such a gene is suitable as a normal form of a gene, however, other forms which provide similar functional characteristics may also be used as a normal gene. In particular, a normal form of a gene does not confer a growth conditional phenotype on the strain having that gene, while a mutant form of a gene suitable for use in these methods does provide such a growth conditional phenotype.

15 As used herein, the terms “ornithine carbamoyltransferase (OCTase)” and “ornithine carbamoyltransferase (OCTase) polypeptide” refer to an enzyme that catalyzes the reversible interconversion of carbamoyl phosphate and L-ornithine with L-citrulline and phosphate. Although the protein and/or the name of the gene that encodes the protein may differ between species, the terms “OCTase” and “ARG3 gene product” are intended 20 to encompass any polypeptide that catalyzes the reversible interconversion of carbamoyl phosphate and L-ornithine with L-citrulline and phosphate. For example, the phrase “OCTase gene” includes the ARG3 gene from *M. grisea* as well as genes from other organisms that encode a polypeptide that catalyzes the reversible interconversion of carbamoyl phosphate and L-ornithine with L-citrulline and phosphate.

25 As used herein, the term “pathogenicity” refers to a capability of causing disease and/or degree of capacity to cause disease. The term is applied to parasitic micro-organisms in relation to their hosts. As used herein, “pathogenicity,” “pathogenic,” and the like, encompass the general capability of causing disease as well as various mechanisms and structural and/or functional deviations from normal used in the art to 30 describe the causative factors and/or mechanisms, presence, pathology, and/or progress of disease, such as virulence, host recognition, cell wall degradation, toxin production,

infection hyphae, penetration peg production, appressorium production, lesion formation, sporulation, and the like.

The “percent (%) sequence identity” between two polynucleotide or two polypeptide sequences is determined according to either the BLAST program (Basic Local Alignment Search Tool, (Altschul, S.F. *et al.*, 215 J. Mol. Biol. 403 (1990) (PMID: 2231712)) or using Smith Waterman Alignment (T.F. Smith & M. S. Waterman 147 J. Mol. Biol. 195 (1981) (PMID: 7265238)). It is understood that for the purposes of determining sequence identity when comparing a DNA sequence to an RNA sequence, a thymine nucleotide is equivalent to a uracil nucleotide.

10 By “polypeptide” is meant a chain of at least two amino acids joined by peptide bonds. The chain may be linear, branched, circular or combinations thereof. The polypeptides may contain amino acid analogs and other modifications, including, but not limited to glycosylated or phosphorylated residues.

As used herein, the term "proliferation" is synonymous to the term "growth."

15 As used herein, "semi-permissive conditions" are conditions in which the relevant culture parameter for a particular growth conditional phenotype is intermediate between permissive conditions and non-permissive conditions. Consequently, in semi-permissive conditions an organism having a growth conditional phenotype will exhibit growth rates intermediate between those shown in permissive conditions and non-permissive conditions. In general, such intermediate growth rate may be due to a mutant cellular component that is partially functional under semi-permissive conditions, essentially fully functional under permissive conditions, and is non-functional or has very low function under non-permissive conditions, where the level of function of that component is related to the growth rate of the organism. An intermediate growth rate may also be a result of a 20 nutrient substance or substances that are present in amounts not sufficient for optimal growth rates to be achieved.

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“Sensitivity phenotype” refers to a phenotype that exhibits either hypersensitivity or hyposensitivity.

30 The term “specific binding” refers to an interaction between OCTase and a molecule or compound, wherein the interaction is dependent upon the primary amino

acid sequence and/or the tertiary conformation of OCTase. An “OCTase ligand” is an example of specific binding.

“Transform,” as used herein, refers to the introduction of a polynucleotide (single or double stranded DNA, RNA, or a combination thereof) into a living cell by any means.

5 Transformation may be accomplished by a variety of methods, including, but not limited to, electroporation, polyethylene glycol mediated uptake, particle bombardment, agrotransformation, and the like. The transformation process may result in transient or stable expression of the transformed polynucleotide. By “stably transformed” is meant that the sequence of interest is integrated into a replicon in the cell, such as a

10 chromosome or episome. Transformed cells encompass not only the end product of a transformation process, but also the progeny thereof which retain the polynucleotide of interest.

For the purposes of the invention, “transgenic” refers to any cell, spore, tissue or part, that contains all or part of at least one recombinant polynucleotide. In many cases,

15 all or part of the recombinant polynucleotide is stably integrated into a chromosome or stable extra-chromosomal element, so that it is passed on to successive generations.

As used herein, the term "Tween 20" means sorbitan mono-9-octadecenoate poly(oxy-1,1-ethanediyl).

As used in this disclosure, the term "viability" of an organism refers to the ability

20 of an organism to demonstrate growth under conditions appropriate for the organism, or to demonstrate an active cellular function. Some examples of active cellular functions include respiration as measured by gas evolution, secretion of proteins and/or other compounds, dye exclusion, mobility, dye oxidation, dye reduction, pigment production, changes in medium acidity, and the like.

25 The present inventors have discovered that disruption of the OCTase gene and/or gene product eliminates the pathogenicity of *Magnaporthe grisea*. Thus, the inventors demonstrate that OCTase is a target for antibiotics, preferably antifungals.

Accordingly, the invention provides methods for identifying compounds that inhibit OCTase gene expression or biological activity of its gene product(s). Such

30 methods include ligand-binding assays, assays for enzyme activity, cell-based assays, and

assays for OCTase gene expression. The compounds identified by the methods of the invention are useful as antibiotics.

Thus, in one embodiment, the invention provides a method for identifying a test compound as a candidate for an antibiotic, comprising contacting an OCTase polypeptide with a test compound and detecting the presence or absence of binding between the test compound and the OCTase polypeptide, wherein binding indicates that the test compound is a candidate for an antibiotic.

OCTase polypeptides of the invention have the amino acid sequence of a naturally occurring OCTase found in a fungus, animal, plant, or microorganism, or have an amino acid sequence derived from a naturally occurring sequence. Preferably the OCTase is a fungal OCTase. A cDNA encoding *M. grisea* OCTase protein is set forth in SEQ ID NO:1, an *M. grisea* OCTase genomic DNA is set forth in SEQ ID NO:2, and an *M. grisea* OCTase polypeptide is set forth in SEQ ID NO:3. In one embodiment, the OCTase is a *Magnaporthe* OCTase. *Magnaporthe* species include, but are not limited to, *Magnaporthe rhizophila*, *Magnaporthe salvinii*, *Magnaporthe grisea* and *Magnaporthe poae* and the imperfect states of *Magnaporthe* in the genus *Pyricularia*. Preferably, the *Magnaporthe* OCTase is from *Magnaporthe grisea*.

In one embodiment, the invention provides a polypeptide consisting essentially of SEQ ID NO:3. For the purposes of the present invention, a polypeptide consisting essentially of SEQ ID NO:3 has at least 90% sequence identity with *M. grisea* OCTase (SEQ ID NO:3) and at least 10% of the activity of SEQ ID NO:3. A polypeptide consisting essentially of SEQ ID NO:3 has at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity with SEQ ID NO:3 and at least 25%, 50%, 75%, or 90% of the activity of *M. grisea* OCTase. Examples of polypeptides consisting essentially of SEQ ID NO:3 include, but are not limited to, polypeptides having the amino acid sequence of SEQ ID NO:3 with the exception that one or more of the amino acids are substituted with structurally similar amino acids providing a conservative amino acid substitution. Conservative amino acid substitutions are well known to those of skill in the art. Examples of polypeptides consisting essentially of SEQ ID NO:3 include polypeptides having 1, 2, or 3 conservative amino acid substitutions relative to SEQ ID NO:3. Other examples of polypeptides consisting essentially of SEQ ID NO:3 include

polypeptides having the sequence of SEQ ID NO:3, but with truncations at either or both the 3' and the 5' end. For example, polypeptides consisting essentially of SEQ ID NO:3 include polypeptides having 1, 2, or 3 amino acids residues removed from either or both 3' and 5' ends relative to SEQ ID NO:3.

5 In various embodiments, the OCTase can be from Powdery Scab (*Spongospora subterranea*), Grey Mould (*Botrytis cinerea*), White Rot (*Armillaria mellea*), Heartrot Fungus (*Ganoderma adspersum*), Brown-Rot (*Piptoporus betulinus*), Corn Smut (*Ustilago maydis*), Heartrot (*Polyporus squamosus*), Gray Leaf Spot (*Cercospora zeae-maydis*), Honey Fungus (*Armillaria gallica*), Root rot (*Armillaria luteobubalina*),  
10 Shoestring Rot (*Armillaria ostoyae*), Banana Anthracnose Fungus (*Colletotrichum musae*), Apple-rotting Fungus (*Monilinia fructigena*), Apple-rotting Fungus (*Penicillium expansum*), Clubroot Disease (*Plasmodiophora brassicae*), Potato Blight (*Phytophthora infestans*), Root pathogen (*Heterobasidion annosum*), Take-all Fungus (*Gaeumannomyces graminis*), Dutch Elm Disease (*Ophiostoma ulmi*), Bean Rust  
15 (*Uromyces appendiculatus*), Northern Leaf Spot (*Cochliobolus carbonum*), Milo Disease (*Periconia circinata*), Southern Corn Blight (*Cochliobolus heterostrophus*), Leaf Spot (*Cochliobolus lunata*), Brown Stripe (*Cochliobolus stenospilus*), Panama disease (*Fusarium oxysporum*), Wheat Head Scab Fungus (*Fusarium graminearum*), Cereal Foot Rot (*Fusarium culmorum*), Potato Black Scurf (*Rhizoctonia solani*), Wheat Black Stem  
20 Rust (*Puccinia graminis*), White mold (*Sclerotinia sclerotiorum*), and the like.

Fragments of an OCTase polypeptide are useful in the methods of the invention. In one embodiment, the OCTase fragments include an intact or nearly intact epitope that occurs on the biologically active wild-type OCTase. For example, the fragments comprise at least 10 consecutive amino acids of the OCTase set forth in SEQ ID NO:3.  
25 The fragments comprise at least 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 125, 150, 175, 200, 225, 250, 275, 300, 325 or at least 345 consecutive amino acids residues of OCTase set forth in SEQ ID NO:3. Fragments of heterologous OCTases are also useful in the methods of the invention. For example, polypeptides having at least 50%, 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity with at least 50  
30 consecutive amino acid residues of SEQ ID NO:2 are useful in the methods of the invention. In one embodiment, the fragment is from a *Magnaporthe* OCTase. In an

alternate embodiment, the fragment contains an amino acid sequence conserved among fungal OCTases.

Polypeptides having at least 40% sequence identity with *M. grisea* OCTase (SEQ ID NO:3) protein are also useful in the methods of the invention. In one embodiment, the sequence identity is at least 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99%, or any integer from 40-100% sequence identity in ascending order with *M. grisea* OCTase (SEQ ID NO:3) protein. In addition, it is preferred that polypeptides of the invention have at least 10% of the activity of *M. grisea* OCTase (SEQ ID NO:3) protein. OCTase polypeptides of the invention have at least 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85% or at least 90% of the activity of *M. grisea* OCTase (SEQ ID NO:3) protein.

Thus, in another embodiment, the invention provides a method for identifying a test compound as a candidate for a fungicide, comprising: contacting a test compound with at least one polypeptide selected from the group consisting of: a polypeptide consisting essentially of SEQ ID NO:3, a polypeptide having at least ten consecutive amino acids of an *M. grisea* OCTase (SEQ ID NO:3) protein, a polypeptide having at least 50% sequence identity with an *M. grisea* OCTase (SEQ ID NO:3) protein and at least 10% of the activity of an *M. grisea* OCTase (SEQ ID NO:3) protein, and a polypeptide consisting of at least 50 amino acids having at least 50% sequence identity with an *M. grisea* OCTase (SEQ ID NO:3) protein and at least 10% of the activity of an *M. grisea* OCTase (SEQ ID NO:3) protein, and detecting the presence and/or absence of binding between the test compound and the polypeptide, wherein binding indicates that the test compound is a candidate for an antibiotic.

Any technique for detecting the binding of a ligand to its target may be used in the methods of the invention. For example, the ligand and target are combined in a buffer. Many methods for detecting the binding of a ligand to its target are known in the art, and include, but are not limited to, the detection of an immobilized ligand-target complex or the detection of a change in the properties of a target when it is bound to a ligand. For example, in one embodiment, an array of immobilized candidate ligands is provided. The immobilized ligands are contacted with an OCTase protein or a fragment or variant

thereof, the unbound protein is removed, and the bound OCTase is detected. In a preferred embodiment, bound OCTase is detected using a labeled binding partner, such as a labeled antibody. In an alternate preferred embodiment, OCTase is labeled prior to contacting the immobilized candidate ligands. Preferred labels include fluorescent or radioactive moieties. Preferred detection methods include fluorescence correlation spectroscopy (FCS) and FCS-related confocal nanofluorimetric methods.

Once a compound is identified as a candidate for an antibiotic, it can be tested for the ability to inhibit OCTase enzymatic activity. The compounds can be tested using either *in vitro* or cell based assays. Alternatively, a compound can be tested by applying it directly to a fungus or fungal cell, or expressing it therein, and monitoring the fungus or fungal cell for changes or decreases in growth, development, viability, pathogenicity, or alterations in gene expression. Thus, in one embodiment, the invention provides a method for determining whether a compound identified as an antibiotic candidate by an above method has antifungal activity, further comprising: contacting a fungus or fungal cells with the antifungal candidate and detecting a decrease in the growth, viability, or pathogenicity of the fungus or fungal cells.

By decrease in growth, is meant that the antifungal candidate causes at least a 10% decrease in the growth of the fungus or fungal cells, as compared to the growth of the fungus or fungal cells in the absence of the antifungal candidate. By a decrease in viability is meant that at least 20% of the fungal cells, or portion of the fungus contacted with the antifungal candidate are nonviable. Preferably, the growth or viability will be decreased by at least 40%. More preferably, the growth or viability will be decreased by at least 50%, 75% or at least 90% or more. Methods for measuring fungal growth and cell viability are known to those skilled in the art. By decrease in pathogenicity, is meant that the antifungal candidate causes at least a 10% decrease in the disease caused by contact of the fungal pathogen with its host, as compared to the disease caused in the absence of the antifungal candidate. Preferably, the disease will be decreased by at least 40%. More preferably, the disease will be decreased by at least 50%, 75% or at least 90% or more. Methods for measuring fungal disease are well known to those skilled in the art, and include such metrics as lesion formation, lesion size, sporulation, respiratory failure, and/or death.

The ability of a compound to inhibit OCTase activity can be detected using *in vitro* enzymatic assays in which the disappearance of a substrate or the appearance of a product is directly or indirectly detected. OCTase catalyzes the reversible interconversion of carbamoyl phosphate and L-ornithine with L-citrulline and phosphate 5 (see Figure 1). Methods for measuring the progression of the OCTase enzymatic reaction and/or a change in the concentration of the individual reactants carbamoyl phosphate, L-ornithine, L-citrulline, and phosphate, include spectrophotometry, fluorimetry, mass spectroscopy, thin layer chromatography (TLC) and reverse phase HPLC.

Thus, the invention provides a method for identifying a test compound as a 10 candidate for an antibiotic, comprising: contacting carbamoyl phosphate and L-ornithine with an OCTase in the presence and absence of a test compound or contacting L-citrulline and phosphate with an OCTase in the presence and absence of a test compound; and determining a change in concentration for at least one of carbamoyl phosphate, L-ornithine, L-citrulline and/or phosphate in the presence and absence of the test 15 compound, wherein a change in the concentration for any of the above reactants indicates that the test compound is a candidate for an antibiotic.

Enzymatically active fragments of *M. grisea* OCTase set forth in SEQ ID NO:3 are also useful in the methods of the invention. For example, an enzymatically active polypeptide comprising at least 50 consecutive amino acid residues and at least 10% of 20 the activity of *M. grisea* OCTase set forth in SEQ ID NO:3 are useful in the methods of the invention. In addition, fragments of heterologous OCTases are also useful in the methods of the invention. Enzymatically active polypeptides having at least 10% of the activity of SEQ ID NO:3 and at least 50%, 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity with at least 50 consecutive amino acid residues of SEQ ID 25 NO:3 are useful in the methods of the invention. Most preferably, the enzymatically active polypeptide has at least 50% sequence identity with at least 50 consecutive amino acid residues of SEQ ID NO:3 and at least 25%, 75% or at least 90% of the activity thereof.

Thus, the invention provides a method for identifying a test compound as a 30 candidate for an antibiotic, comprising: contacting carbamoyl phosphate and L-ornithine or L-citrulline and phosphate with a polypeptide selected from the group consisting of: a

polypeptide consisting essentially of SEQ ID NO:3, a polypeptide having at least 50% sequence identity with the *M. grisea* OCTase set forth in SEQ ID NO:3 and having at least 10% of the activity thereof, a polypeptide comprising at least 50 consecutive amino acids of *M. grisea* OCTase set forth in SEQ ID NO:3 and having at least 10% of the activity thereof, and a polypeptide consisting of at least 50 amino acids and having at least 50% sequence identity with *M. grisea* OCTase set forth in SEQ ID NO:3 and having at least 10% of the activity thereof, contacting carbamoyl phosphate and L-ornithine or L-citrulline and phosphate with the polypeptide and a test compound, and determining a change in concentration for at least one of carbamoyl phosphate, L-ornithine, L-citrulline and/or phosphate in the presence and absence of the test compound, wherein a change in concentration for any of the above substances indicates that the test compound is a candidate for an antibiotic.

For *in vitro* enzymatic assays, OCTase protein and derivatives thereof may be isolated from a fungus or may be recombinantly produced in and isolated from an archael, bacterial, fungal, or other eukaryotic cell culture. Preferably these proteins are produced using an *E. coli*, yeast, or filamentous fungal expression system. Methods for the purification of OCTase proteins and polypeptides are known to those skilled in the art.

As an alternative to *in vitro* assays, the invention also provides cell based assays. In one embodiment, the invention provides a method for identifying a test compound as a candidate for an antibiotic, comprising: a) measuring the expression or activity of an OCTase in a cell, cells, tissue, or an organism in the absence of a test compound; b) contacting the cell, cells, tissue, or organism with the test compound and measuring the expression or activity of the OCTase in the cell, cells, tissue, or organism; and c) comparing the expression or activity of the OCTase in steps (a) and (b), wherein an altered expression or activity in the presence of the test compound indicates that the compound is a candidate for an antibiotic.

Expression of OCTase can be measured by detecting the OCTase primary transcript or mRNA, OCTase polypeptide, or OCTase enzymatic activity. Methods for detecting the expression of RNA and proteins are known to those skilled in the art.

(*Current Protocols in Molecular Biology*, Ausubel *et al.*, eds., Greene Publishing &

Wiley-Interscience, New York, (1995)). The method of detection is not critical to the present invention. Methods for detecting OCTase RNA include, but are not limited to, amplification assays such as quantitative reverse transcriptase-PCR, and/or hybridization assays such as Northern analysis, dot blots, slot blots, in-situ hybridization,  
5 transcriptional fusions using an OCTase promoter fused to a reporter gene, DNA assays, and microarray assays.

Methods for detecting protein expression include, but are not limited to, immunodetection methods such as Western blots, ELISA assays, polyacrylamide gel electrophoresis, mass spectroscopy, and enzymatic assays. Also, any reporter gene  
10 system may be used to detect OCTase protein expression. For detection using gene reporter systems, a polynucleotide encoding a reporter protein is fused in frame with OCTase so as to produce a chimeric polypeptide. Methods for using reporter systems are known to those skilled in the art.

Chemicals, compounds, or compositions identified by the above methods as  
15 modulators of OCTase expression or activity can then be used to control fungal growth. Diseases such as rusts, mildews, and blights spread rapidly once established. Fungicides are thus routinely applied to growing and stored crops as a preventive measure, generally as foliar sprays or seed dressings. For example, compounds that inhibit fungal growth can be applied to a fungus or expressed in a fungus to prevent fungal growth. Thus, the  
20 invention provides a method for inhibiting fungal growth, comprising contacting a fungus with a compound identified by the methods of the invention as having antifungal activity.

Antifungals and antifungal inhibitor candidates identified by the methods of the invention can be used to control the growth of undesired fungi, including ascomycota, zygomycota, basidiomycota, chytridiomycota, and lichens. Examples of undesired fungi  
25 include, but are not limited to Powdery Scab (*Spongospora subterranea*), Grey Mould (*Botrytis cinerea*), White Rot (*Armillaria mellea*), Heartrot Fungus (*Ganoderma adspersum*), Brown-Rot (*Piptoporus betulinus*), Corn Smut (*Ustilago maydis*), Heartrot (*Polyporus squamosus*), Gray Leaf Spot (*Cercospora zeae-maydis*), Honey Fungus (*Armillaria gallica*), Root rot (*Armillaria luteobubalina*), Shoestring Rot (*Armillaria ostoyae*), Banana Anthracnose Fungus (*Colletotrichum musae*), Apple-rotting Fungus (*Monilinia fructigena*), Apple-rotting Fungus (*Penicillium expansum*), Clubroot Disease

(*Plasmodiophora brassicae*), Potato Blight (*Phytophthora infestans*), Root pathogen (*Heterobasidion annosum*), Take-all Fungus (*Gaeumannomyces graminis*), Dutch Elm Disease (*Ophiostoma ulmi*), Bean Rust (*Uromyces appendiculatus*), Northern Leaf Spot (*Cochliobolus carbonum*), Milo Disease (*Periconia circinata*), Southern Corn Blight 5 (*Cochliobolus heterostrophus*), Leaf Spot (*Cochliobolus lunata*), Brown Stripe (*Cochliobolus stenospilus*), Panama disease (*Fusarium oxysporum*), Wheat Head Scab Fungus (*Fusarium graminearum*), Cereal Foot Rot (*Fusarium culmorum*), Potato Black Scurf (*Rhizoctonia solani*), Wheat Black Stem Rust (*Puccinia graminis*), White mold 10 (*Sclerotinia sclerotiorum*), diseases of animals such as infections of lungs, blood, brain, skin, scalp, nails or other tissues (*Aspergillus fumigatus* *Aspergillus* sp. *Fusarium* sp., *Trichophyton* sp., *Epidermophyton* sp., and *Microsporum* sp., and the like).

Also provided in the invention are methods of screening for an antibiotic by determining the *in vivo* activity of a test compound against two separate fungal organisms, wherein the fungal organisms comprise a first form of an OCTase and a 15 second form of the OCTase, respectively. In the methods of the invention, at least one of the two forms of the OCTase has at least 10% of the activity of the polypeptide set forth in SEQ ID NO:3. The methods comprise comparing the growth of the two organisms in the presence of the test compound relative to their respective controls without the test compound. A difference in growth between the two organisms in the presence of the test 20 compound indicates that the test compound is a candidate for an antibiotic.

Forms of an OCTase useful in the methods of the invention are selected from the group consisting of: a nucleic acid encoding SEQ ID NO:3, a nucleic acid encoding a polypeptide consisting essentially of SEQ ID NO:3, SEQ ID NO:1 or SEQ ID NO:2, SEQ ID NO:1 or SEQ ID NO:2 comprising a mutation either reducing or abolishing 25 OCTase protein activity, a heterologous OCTase, and a heterologous OCTase comprising a mutation either reducing or abolishing OCTase protein activity. Any combination of two different forms of the OCTase genes listed above are useful in the methods of the invention, with the caveat that at least one of the forms of the OCTase has at least 10% of the activity of the polypeptide set forth in SEQ ID NO:3.

Thus, in one embodiment, the invention provides a method for identifying a test compound as a candidate for an antibiotic, comprising: providing an organism having a

first form of an OCTase; providing an organism having a second form of the OCTase; and determining the growth of the organism having the first form of the OCTase and the growth of the organism having the second form of the OCTase in the presence of the test compound, wherein a difference in growth between the two organisms in the presence of  
5 the test compound indicates that the test compound is a candidate for an antibiotic. It is recognized in the art that the optional determination of the growth of the organism having the first form of the OCTase and the growth of the organism having the second form of the OCTase in the absence of any test compounds is performed to control for any inherent differences in growth as a result of the different genes. Growth and/or  
10 proliferation of an organism are measured by methods well known in the art such as optical density measurements, and the like. In a preferred embodiment, the organism is *Magnaporthe grisea*.

In another embodiment, the invention provides a method for identifying a test compound as a candidate for an antibiotic, comprising: providing an organism having a  
15 first form of an OCTase; providing a comparison organism having a second form of the OCTase; and determining the pathogenicity of the organism having the first form of the OCTase and the organism having the second form of the OCTase in the presence of the test compound, wherein a difference in pathogenicity between the two organisms in the presence of the test compound indicates that the test compound is a candidate for an  
20 antibiotic. In an alternate embodiment of the invention, the pathogenicity of the organism having the first form of the OCTase and the organism having the second form of the OCTase in the absence of any test compounds is determined to control for any inherent differences in pathogenicity as a result of the different genes. Pathogenicity of an organism is measured by methods well known in the art such as lesion number, lesion  
25 size, sporulation, and the like. In a preferred embodiment the organism is *Magnaporthe grisea*.

In one embodiment of the invention, the first form of an OCTase is SEQ ID NO:1 or SEQ ID NO:2, and the second form of the OCTase is an OCTase that confers a growth conditional phenotype (*i.e.* an arginine requiring phenotype) and/or a hypersensitivity or  
30 hyposensitivity phenotype on the organism. In a related embodiment of the invention, the second form of the OCTase is SEQ ID NO:1 comprising a transposon insertion that

reduces activity. In still another embodiment of the invention, the second form of a OCTase is SEQ ID NO:1 comprising a transposon insertion that abolishes activity. In a related embodiment of the invention, the second form of the OCTase is SEQ ID NO:2 comprising a transposon insertion that reduces activity. In a further embodiment of the  
5 invention, the second form of the OCTase is SEQ ID NO:2 comprising a transposon insertion that abolishes activity. In yet another embodiment of the invention, the second form of the OCTase is *N. crassa* OCTase. In a related embodiment of the invention, the second form of the OCTase is *A. terreus* OCTase.

In another embodiment of the invention, the first form of an OCTase is *N. crassa*  
10 OCTase and the second form of the OCTase is *N. crassa* OCTase comprising a transposon insertion that reduces activity. In a related embodiment of the invention, the second form of the OCTase is *N. crassa* OCTase comprising a transposon insertion that abolishes activity. In another embodiment of the invention, the first form of an OCTase is *A. terreus* OCTase and the second form of the OCTase is *A. terreus* OCTase  
15 comprising a transposon insertion that reduces activity. In a related embodiment of the invention, the second form of the OCTase is *A. terreus* OCTase comprising a transposon insertion that abolishes activity.

Conditional lethal mutants and/or antipathogenic mutants identify particular biochemical and/or genetic pathways given that at least one identified target gene is  
20 present in that pathway. Knowledge of these pathways allows for the screening of test compounds as candidates for antibiotics as inhibitors of the substrates, products, proteins and/or enzymes of the pathway. The invention provides methods of screening for an antibiotic by determining whether a test compound is active against the arginine biosynthetic pathway on which OCTase functions. Pathways known in the art are found  
25 at the Kyoto Encyclopedia of Genes and Genomes and in standard biochemistry texts (See, e.g. Lehninger *et al.*, Principles of Biochemistry, New York, Worth Publishers (1993)).

Thus, in one embodiment, the invention provides a method for screening for test compounds acting against the biochemical and/or genetic pathway or pathways in which  
30 OCTase functions, comprising: providing an organism having a first form of a gene in the arginine biosynthetic pathway; providing an organism having a second form of the gene

in the arginine biosynthetic pathway; and determining the growth of the two organisms in the presence of a test compound, wherein a difference in growth between the organism having the first form of the gene and the organism having the second form of the gene in the presence of the test compound indicates that the test compound is a candidate for an antibiotic. It is recognized in the art that the optional determination of the growth of the organism having the first form of the gene and the organism having the second form of the gene in the absence of any test compounds is performed to control for any inherent differences in growth as a result of the different genes. Growth and/or proliferation of an organism are measured by methods well known in the art, such as optical density

10 measurements and the like. In a preferred embodiment, the organism is *Magnaporthe grisea*.

The forms of a gene in the arginine biosynthetic pathway useful in the methods of the invention include, for example, wild-type and mutated genes encoding argininosuccinate lyase and argininosuccinate synthase from any organism, preferably from a fungal organism, and most preferably from *M. grisea*. The forms of a mutated gene in the arginine biosynthetic pathway comprise a mutation either reducing or abolishing protein activity. In one example, the form of a gene in the arginine biosynthetic pathway comprises a transposon insertion. Any combination of a first form of a gene in the arginine biosynthetic pathway and a second form of the gene listed above

15 are useful in the methods of the invention, with the limitation that one of the forms of a gene in the arginine biosynthetic pathway has at least 10% of the activity of the corresponding *M. grisea* gene.

In another embodiment, the invention provides a method for screening for test compounds acting against the biochemical and/or genetic pathway or pathways in which

20 OCTase functions, comprising: providing an organism having a first form of a gene in the arginine biosynthetic pathway; providing an organism having a second form of the gene in the arginine biosynthetic pathway; and determining the pathogenicity of the two organisms in the presence of the test compound, wherein a difference in pathogenicity between the organism having the first form of the gene and the organism having the second form of the gene in the presence of the test compound indicates that the test

25 compound is a candidate for an antibiotic. In an optional embodiment of the invention,

the pathogenicity of the two organisms in the absence of any test compounds is determined to control for any inherent differences in pathogenicity as a result of the different genes. Pathogenicity of an organism is measured by methods well known in the art such as lesion number, lesion size, sporulation, and the like. In a preferred 5 embodiment the organism is *Magnaporthe grisea*.

Thus, in an alternate embodiment, the invention provides a method for screening for test compounds acting against the biochemical and/or genetic pathway or pathways in which OCTase functions, comprising: providing paired growth media containing a test compound, wherein the paired growth media comprise a first medium and a second 10 medium and the second medium contains a higher level of L-arginine than the first medium; inoculating the first and the second medium with an organism; and determining the growth of the organism, wherein a difference in growth of the organism between the first and the second medium indicates that the test compound is a candidate for an antibiotic. In one embodiment of the invention, the growth of the organism is determined 15 in the first and the second medium in the absence of any test compounds to control for any inherent differences in growth as a result of the different media. Growth and/or proliferation of the organism are measured by methods well known in the art such as optical density measurements, and the like. In a preferred embodiment, the organism is *Magnaporthe grisea*.

One embodiment of the invention is directed to the use of multi-well plates for 20 screening of antibiotic compounds. The use of multi-well plates is a format that readily accommodates multiple different assays to characterize various compounds, concentrations of compounds, and fungal organisms in varying combinations and formats. Certain testing parameters for the screening method can significantly affect the 25 identification of growth inhibitors, and thus can be manipulated to optimize screening efficiency and/or reliability. Notable among these factors are variable sensitivities of different mutants, increasing hypersensitivity with increasingly less permissive conditions, an apparent increase in hypersensitivity with increasing compound concentration, and other factors known to those in the art.

30

## EXPERIMENTAL

### Example 1

#### Construction of Plasmids with a Transposon Containing a Selectable Marker.

##### Construction of Sif transposon:

Sif was constructed using the GPS3 vector from the GPS-M mutagenesis system  
5 from New England Biolabs, Inc. (Beverly, MA) as a backbone. This system is based on  
the bacterial transposon Tn7. The following manipulations were done to GPS3 according  
to Sambrook *et al.*, Molecular Cloning, a Laboratory Manual, Cold Spring Harbor  
Laboratory Press (1989). The kanamycin resistance gene (npt) contained between the  
Tn7 arms was removed by EcoRV digestion. The bacterial hygromycin B  
10 phosphotransferase (hph) gene (Gritz & Davies, 25 Gene 179 (1983) (PMID: 6319235))  
under control of the *Aspergillus nidulans* trpC promoter and terminator (Mullaney *et al.*,  
199 Mol. Gen. 37 (1985) (PMID: 3158796)) was cloned by a HpaI/EcoRV blunt  
ligation into the Tn7 arms of the GPS3 vector yielding pSif1. Excision of the ampicillin  
resistance gene (bla) from pSif1 was achieved by cutting pSif1 with XmnI and BglII  
15 followed by a T4 DNA polymerase treatment to remove the 3' overhangs left by the BglII  
digestion and religation of the plasmid to yield pSif. Top 10F' electrocompetent *E. coli*  
cells (Invitrogen) were transformed with ligation mixture according to manufacturer's  
recommendations. Transformants containing the Sif transposon were selected on LB  
agar (Sambrook *et al.*, *supra*) containing 50 µg/ml of hygromycin B (Sigma Chem. Co.,  
20 St. Louis, MO).

### Example 2

#### Construction of a Fungal Cosmid Library

Cosmid libraries were constructed in the pcosKA5 vector (Hamer *et al.*, 98 Proc.  
25 Nat'l. Acad. Sci. USA 5110 (2001) (PMID: 11296265)) as described in Sambrook *et al.*  
Cosmid libraries were quality checked by pulsed-field gel electrophoresis, restriction  
digestion analysis, and PCR identification of single genes.

### Example 3

#### Construction of Cosmids with Transposon Insertion into Fungal Genes

Sif Transposition into a Cosmid:

Transposition of Sif into the cosmid framework was carried out as described by the GPS-M mutagenesis system (New England Biolabs, Inc.). Briefly, 2 µl of the 10X GPS buffer, 70 ng of supercoiled pSIF, 8-12 µg of target cosmid DNA were mixed and taken to a final volume of 20 µl with water. 1 µl of transposase (TnsABC) was added to 5 the reaction and incubated for 10 minutes at 37°C to allow the assembly reaction to occur. After the assembly reaction, 1 µl of start solution was added to the tube, mixed well, and incubated for 1 hour at 37°C followed by heat inactivation of the proteins at 75°C for 10 minutes. Destruction of the remaining untransposed pSif was completed by PISceI digestion at 37°C for 2 hours followed by a 10 minute incubation at 75°C to inactivate the 10 proteins. Transformation of Top10F' electrocompetent cells (Invitrogen) was done according to manufacturers recommendations. Sif-containing cosmid transformants were selected by growth on LB agar plates containing 50 µg/ml of hygromycin B (Sigma Chem. Co.) and 100 µg/ml of Ampicillin (Sigma Chem. Co.).

15

#### Example 4

##### High Throughput Preparation and Verification of Transposon Insertion into the *M. grisea* ARG3 Gene

*E. coli* strains containing cosmids with transposon insertions were picked to 96 well growth blocks (Beckman Co.) containing 1.5 ml of TB (Terrific Broth, Sambrook *et al.*, *supra*) supplemented with 50 µg/ml of ampicillin. Blocks were incubated with 20 shaking at 37°C overnight. *E. coli* cells were pelleted by centrifugation and cosmids were isolated by a modified alkaline lysis method (Marra *et al.*, 7 Genome Res. 1072 (1997) (PMID: 9371743)). DNA quality was checked by electrophoresis on agarose gels. Cosmids were sequenced using primers from the ends of each transposon and commercial 25 dideoxy sequencing kits (Big Dye Terminators, Perkin Elmer Co.). Sequencing reactions were analyzed on an ABI377 DNA sequencer (Perkin Elmer Co.).

The DNA sequences adjacent to the site of the transposon insertion were used to search DNA and protein databases using the BLAST algorithms (Altschul *et al.*, *supra*). A single insertion of SIF into the *Magnaporthe grisea* ARG3 gene was chosen for further 30 analysis. This construct was designated cpgmra0049002a06 and it contains the SIF transposon insertion within the OCTase coding region.

### Example 5

#### Preparation of ARG3 Cosmid DNA and Transformation of *Magnaporthe grisea*

Cosmid DNA from the ARG3 transposon tagged cosmid clone was prepared  
5 using QIAGEN Plasmid Maxi Kit (Qiagen), and digested by PI-PspI (New England  
Biolabs, Inc.). Fungal electro-transformation was performed essentially as described  
(Wu *et al.*, 10 MPMI 700 (1997)). Briefly, *M. grisea* strain Guy 11 was grown in  
complete liquid media (Talbot *et al.*, 5 Plant Cell 1575 (1993) (PMID: 8312740)) shaking  
at 120 rpm for 3 days at 25°C in the dark. Mycelia was harvested and washed with sterile  
10 H<sub>2</sub>O and digested with 4 mg/ml beta-glucanase (InterSpex) for 4-6 hours to generate  
protoplasts. Protoplasts were collected by centrifugation and resuspended in 20% sucrose  
at a concentration of 2x10<sup>8</sup> protoplasts/ml. 50 µl of protoplast suspension was mixed  
with 10-20 µg of the cosmid DNA and pulsed using a Gene Pulser II instrument (BioRad)  
set with the following parameters: 200 ohm, 25µF, and 0.6kV. Transformed protoplasts  
15 were regenerated in complete agar media (Talbot *et al.*, *supra*) with the addition of 20%  
sucrose for one day, then overlayed with CM agar media containing hygromycin B (250  
ug/ml) to select transformants. Transformants were screened for homologous  
recombination events in the target gene by PCR (Hamer *et al.*, *supra*). Two independent  
strains were identified and are hereby referred to as KO1-15 and KO1-23, respectively.  
20

### Example 6

#### Effect of Transposon Insertion on *Magnaporthe* pathogenicity

The target fungal strains, KO1-15 and KO1-23, obtained in Example 5 and the  
wild-type strain, Guy11, were subjected to a pathogenicity assay to observe infection  
25 over a 1-week period. Rice infection assays were performed using Indica rice cultivar  
CO39 essentially as described in Valent *et al.* (Valent *et al.*, 127 *Genetics* 87 (1991)  
(PMID: 2016048)). All three strains were grown for spore production on complete agar  
media. Spores were harvested and the concentration of spores adjusted for whole plant  
inoculations. Two-week-old seedlings of cultivar CO39 were sprayed with 12 ml of  
30 conidial suspension (5 x 10<sup>4</sup> conidia per ml in 0.01% Tween-20 solution). The inoculated  
plants were incubated in a dew chamber at 27°C in the dark for 36 hours, and transferred

to a growth chamber ( $27^{\circ}\text{C}$  12 hours/ $21^{\circ}\text{C}$  12 hours at 70% humidity) for an additional 5.5 days. Leaf samples were taken at 3, 5, and 7 days post-inoculation and examined for signs of successful infection (*i.e.* lesions). Figure 2 shows the effects of ARG3 gene disruption on *Magnaporthe* infection at five days post-inoculation.

5

#### Example 7

##### Verification of ARG3 Gene Function by Analysis of Nutritional Requirements

The fungal strains, KO1-15 and KO1-23, containing the ARG3 disrupted gene obtained in Example 5 were analyzed for their nutritional requirement for arginine.

10 Spores for each strain were harvested into minimal media and minimal media containing 4 mM L-arginine (Talbot *et al.*, *supra*). The spore concentrations were adjusted to  $2 \times 10^5$  spores/ml. 200  $\mu\text{l}$  of spore suspension were deposited into wells of a microtiter plate. The plate was incubated at  $25^{\circ}\text{C}$  for 7 days. Optical density (OD) measurements at 600nm were taken daily. Data confirming the annotated gene function is presented as a graph of Turbidity vs. Time showing both the mutant fungi and the wild-type control in the absence (Figure 3A) and presence (Figure 3B) of arginine.

15

#### Example 8

##### Cloning, Expression, and Purification of Ornithine Carbamoyltransferase

20 The following is a protocol to obtain an isolated ornithine carbamoyltransferase protein.

##### Cloning and expression strategies:

An OCTase encoding nucleic acid is cloned into *E. coli* (pET vectors-Novagen), Baculovirus (Pharmingen) and Yeast (Invitrogen) expression vectors containing His/fusion protein tags, and the expression of recombinant protein is evaluated by SDS-PAGE and Western blot analysis.

##### Extraction:

Extract recombinant protein from 250 ml cell pellet in 3 ml of extraction buffer

by sonicating 6 times, with 6 second pulses at 4°C. Centrifuge extract at 15000xg for 10 minutes and collect supernatant. Assess biological activity of the recombinant protein by activity assay.

5     Purification:

Purify recombinant protein by Ni-NTA affinity chromatography (Qiagen).

Purification protocol (perform all steps at 4°C):

- Use 3 ml Ni-beads
- Equilibrate column with the buffer
- 10     • Load protein extract
- Wash with the equilibration buffer
- Elute bound protein with 0.5 M imidazole

Example 9

15     Assays for Measuring Binding of Test Compounds to Ornithine Carbamoyltransferase

The following is a protocol to identify test compounds that bind to the ornithine carbamoyltransferase protein.

- Isolated full-length OTCase polypeptide with a His/fusion protein tag (Example 8) is bound to a HISGRAB Nickel Coated Plate (Pierce, Rockford, IL) following manufacturer's instructions.
- Buffer conditions are optimized (*e.g.* ionic strength or pH, Shoolingin-Jordan *et al.* (1997) Methods Enzymol 281: 309 - 16 (PMID: 9250995)) for binding of radiolabeled carbamoyl phosphate, L-ornithine, L-citrulline or phosphate to the bound ornithine carbamoyltransferase.
- 20     • Screening of test compounds is performed by adding test compound and radioactive carbamoyl phosphate, L-ornithine, L-citrulline or phosphate to the wells of the HISGRAB plate containing bound ornithine carbamoyltransferase.
- The wells are washed to remove excess labeled ligand and scintillation fluid (SCINTIVERSE, Fisher Scientific) is added to each well.
- 30     • The plates are read in a microplate scintillation counter.

- Candidate compounds are identified as wells with lower radioactivity as compared to control wells with no test compound added.

Additionally, an isolated polypeptide comprising 10-50 amino acids from the *M. grisea* ornithine carbamoyltransferase is screened in the same way. A polypeptide 5 comprising 10-50 amino acids is generated by subcloning a portion of the OCTase encoding nucleic acid into a protein expression vector that adds a His-Tag when expressed (see Example 8). Oligonucleotide primers are designed to amplify a portion of the OCTase coding region using the polymerase chain reaction amplification method. The DNA fragment encoding a polypeptide of 10 - 50 amino acids is cloned into an 10 expression vector, expressed in a host organism and isolated as described in Example 8 above.

Test compounds that bind OCTase polypeptide are further tested for antibiotic activity. *M. grisea* is grown as described for spore production on oatmeal agar media (Talbot *et al.*, *supra*). Spores are harvested into minimal media to a concentration of  $2 \times 15 10^5$  spores/ml and the culture is divided. *Id.* The test compound is added to one culture to a final concentration of 20-100  $\mu\text{g}/\text{ml}$ . Solvent only is added to the second culture. The plates are incubated at 25°C for seven days and optical density measurements at 590 nm are taken daily. The growth curves of the solvent control sample and the test compound sample are compared. A test compound is an antibiotic candidate if the 20 growth of the culture containing the test compound is less than the growth of the control culture.

Test compounds that bind OCTase polypeptide are further tested for antipathogenic activity. *M. grisea* is grown as described for spore production on oatmeal agar media (Talbot *et al.*, *supra*). Spores are harvested into water with 0.01% Tween 20 25 to a concentration of  $5 \times 10^4$  spores/ml and the culture is divided. *Id.* The test compound is added to one culture to a final concentration of 20-100  $\mu\text{g}/\text{ml}$ . Solvent only is added to the second culture. Rice infection assays are performed using Indica rice cultivar CO39 essentially as described in Valent *et al.*, *supra*). Two-week-old seedlings of cultivar CO39 are sprayed with 12 ml of conidial suspension. The inoculated plants are incubated 30 in a dew chamber at 27°C in the dark for 36 hours, and transferred to a growth chamber (27 °C 12 hours/21 °C 12 hours at 70% humidity) for an additional 5.5 days. Leaf

samples are examined at 5 days post-inoculation to determine the extent of pathogenicity as compared to the control samples.

Alternatively, antipathogenic activity can be assessed using an excised leaf pathogenicity assay. Spore suspensions are prepared in water only to a concentration of 5  $5 \times 10^4$  spores/ml and the culture is divided. The test compound is added to one culture to a final concentration of 20-100  $\mu\text{g}/\text{ml}$ . Solvent only is added to the second culture. Detached leaf assays are performed by excising 1 cm segments of rice leaves from Indica rice cultivar CO39 and placing them on 1% agarose in water. 10  $\mu\text{l}$  of each spore suspension is placed on the leaf segments and the samples are incubated at 25°C for 5 days in the dark. Leaf samples are examined at 5 days post-inoculation to determine the extent of pathogenicity as compared to the control samples.

#### Example 10

##### Assays for Testing Inhibitors or Candidates for Inhibition of Ornithine

##### Carbamoyltransferase Activity

The enzymatic activity of ornithine carbamoyltransferase is determined in the presence and absence of candidate compounds in a suitable reaction mixture. Candidate compounds are identified by a decrease in products or a lack of a decrease in substrates in the presence of the compound, with the reaction proceeding in either direction.

Candidate compounds are additionally determined in the same manner using a polypeptide comprising a fragment of the *M. grisea* ornithine carbamoyltransferase. The OCTase polypeptide fragment is generated by subcloning a portion of the OCTase encoding nucleic acid into a protein expression vector that adds a His-Tag when expressed (see Example 8). Oligonucleotide primers are designed to amplify a portion of the OCTase coding region using polymerase chain reaction amplification method. The DNA fragment encoding the OCTase polypeptide fragment is cloned into an expression vector, expressed and isolated as described in Example 8 above.

Test compounds identified as inhibitors of OCTase activity are further tested for antibiotic activity. *Magnaporthe grisea* fungal cells are grown under standard fungal growth conditions that are well known and described in the art. *M. grisea* is grown as described for spore production on oatmeal agar media (Talbot *et al., supra*). Spores are

harvested into minimal media to a concentration of  $2 \times 10^5$  spores/ml and the culture is divided. *Id.* The test compound is added to one culture to a final concentration of 20-100 µg/ml. Solvent only is added to the second culture. The plates are incubated at 25°C for seven days and optical density measurements at 590nm are taken daily. The growth 5 curves of the solvent control sample and the test compound sample are compared. A test compound is an antibiotic candidate if the growth of the culture containing the test compound is less than the growth of the control culture.

Test compounds identified as inhibitors of OCTase activity are further tested for antipathogenic activity. *M. grisea* is grown as described for spore production on oatmeal 10 agar media (Talbot *et al.*, *supra*). Spores are harvested into water with 0.01% Tween 20 to a concentration of  $5 \times 10^4$  spores/ml and the culture is divided. *Id.* The test compound is added to one culture to a final concentration of 20-100 µg/ml. Solvent only is added to the second culture. Rice infection assays are performed using Indica rice cultivar CO39 essentially as described in Valent *et al.*, *supra*. Two-week-old seedlings of cultivar CO39 15 are sprayed with 12 ml of conidial suspension. The inoculated plants are incubated in a dew chamber at 27°C in the dark for 36 hours, and transferred to a growth chamber (27 °C 12 hours/21 °C 12 hoursat 70% humidity) for an additional 5.5 days. Leaf samples are examined at 5 days post-inoculation to determine the extent of pathogenicity as compared to the control samples.

20 Alternatively, antipathogenic activity is assessed using an excised leaf pathogenicity assay. Spore suspensions are prepared in water only to a concentration of  $5 \times 10^4$  spores/ml and the culture is divided. The test compound is added to one culture to a final concentration of 20-100 µg/ml. Solvent only is added to the second culture. Detached leaf assays are performed by excising 1 cm segments of rice leaves from Indica 25 rice cultivar CO39 and placing them on 1% agarose in water. 10 µl of each spore suspension is place on the leaf segments and the samples are incubated at 25°C for 5 days in the dark. Leaf samples are examined at 5 days post-inoculation to determine the extent of pathogenicity as compared to the control samples.

Assays for Testing Compounds for Alteration of Ornithine Carbamoyltransferase Gene Expression

*Magnaporthe grisea* fungal cells are grown under standard fungal growth conditions that are well known and described in the art. Wild-type *M. grisea* spores are harvested from cultures grown on complete agar or oatmeal agar media after growth for 10-13 days in the light at 25°C using a moistened cotton swab. The concentration of spores is determined using a hemacytometer and spore suspensions are prepared in a minimal growth medium to a concentration of  $2 \times 10^5$  spores per ml. 25 ml cultures are prepared to which test compounds will be added at various concentrations. A culture with no test compound present is included as a control. The cultures are incubated at 25°C for 3 days after which test compound or solvent only control is added. The cultures are incubated an additional 18 hours. Fungal mycelia is harvested by filtration through Miracloth (CalBiochem, La Jolla, CA), washed with water, and frozen in liquid nitrogen. Total RNA is extracted with TRIZOL Reagent using the methods provided by the manufacturer (Life Technologies, Rockville, MD). Expression is analyzed by Northern analysis of the RNA samples as described (Sambrook *et al.*, *supra*) using a radiolabeled fragment of the OCTase encoding nucleic acid as a probe. Test compounds resulting in an altered level of OCTase mRNA relative to the untreated control sample are identified as candidate antibiotic compounds.

Test compounds identified as inhibitors of OCTase expression are further tested for antibiotic activity. *Magnaporthe grisea* fungal cells are grown under standard fungal growth conditions that are well known and described in the art. *M. grisea* is grown as described for spore production on oatmeal agar media (Talbot *et al.*, *supra*). Spores are harvested into minimal media to a concentration of  $2 \times 10^5$  spores/ml and the culture is divided. *Id.* The test compound is added to one culture to a final concentration of 20-100 µg/ml. Solvent only is added to the second culture. The plates are incubated at 25°C for seven days and optical density measurements at 590nm are taken daily. The growth curves of the solvent control sample and the test compound sample are compared. A test compound is an antibiotic candidate if the growth of the culture containing the test compound is less than the growth of the control culture.

Test compounds identified as inhibitors of ARG3 gene expression are further tested for antipathogenic activity. *M. grisea* is grown as described for spore production on oatmeal agar media (Talbot *et al.*, *supra*). Spores are harvested into water with 0.01% Tween 20 to a concentration of  $5 \times 10^4$  spores/ml and the culture is divided. *Id.* The test compound is added to one culture to a final concentration of 20-100 µg/ml. Solvent only is added to the second culture. Rice infection assays are performed using Indica rice cultivar CO39 essentially as described in Valent *et al.*, *supra*. Two-week-old seedlings of cultivar CO39 are sprayed with 12 ml of conidial suspension. The inoculated plants are incubated in a dew chamber at 27°C in the dark for 36 hours, and transferred to a growth chamber (27 °C 12 hours/21 °C 12 hours at 70% humidity) for an additional 5.5 days. Leaf samples are examined at 5 days post-inoculation to determine the extent of pathogenicity as compared to the control samples.

Alternatively, antipathogenic activity is assessed using an excised leaf pathogenicity assay. Spore suspensions are prepared in water only to a concentration of  $5 \times 10^4$  spores/ml and the culture is divided. The test compound is added to one culture to a final concentration of 20-100 µg/ml. Solvent only is added to the second culture. Detached leaf assays are performed by excising 1 cm segments of rice leaves from Indica rice cultivar CO39 and placing them on 1% agarose in water. 10 µl of each spore suspension is placed on the leaf segments and the samples are incubated at 25°C for 5 days in the dark. Leaf samples are examined at 5 days post-inoculation to determine the extent of pathogenicity as compared to the control samples.

#### Example 12

In Vivo Cell Based Assay Screening Protocol with a Fungal Strain Containing a Mutant Form of Ornithine Carbamoyltransferase that Lacks Activity

The effect of test compounds on the growth of wild-type fungal cells and mutant fungal cells having a mutant OCTase gene is measured and compared as follows. *Magnaporthe grisea* fungal cells containing a mutant form of the OCTase gene that lacks activity, for example an OCTase gene containing a transposon insertion, are grown under standard fungal growth conditions that are well known and described in the art.

*Magnaporthe grisea* spores are harvested from cultures grown on complete agar medium

containing L-arginine (Sigma) after growth for 10-13 days in the light at 25°C using a moistened cotton swab. The concentration of spores is determined using a hemacytometer and spore suspensions are prepared in a minimal growth medium containing L-arginine to a concentration of  $2 \times 10^5$  spores per ml. Approximately  $4 \times 10^4$   
5 spores are added to each well of 96-well plates to which a test compound is added (at varying concentrations). The total volume in each well is 200µl. Wells with no test compound present (growth control), and wells without cells are included as controls (negative control). The plates are incubated at 25°C for seven days and optical density measurements at 590nm are taken daily. Wild-type cells are screened under the same  
10 conditions.

The effect of each of the test compounds on the mutant and wild-type fungal cells is measured against the growth control and the percent of inhibition is calculated as the  $OD_{590}$  (fungal strain plus test compound)/ $OD_{590}$  (growth control) x 100. The percent of growth inhibition in the presence of the test compound on the mutant and wild-type  
15 fungal strains are compared. Compounds that show differential growth inhibition between the mutant and the wild-type cells are identified as potential antifungal compounds. Similar protocols may be found in Kirsch & DiDomenico, 26 Biotechnology 177 (1994) (PMID: 7749303)).

Test compounds that produce a differential growth response between the mutant  
20 and wild-type fungal strains are further tested for antipathogenic activity. Each *M. grisea* strain is grown as described for spore production on oatmeal agar media (Talbot *et al.*, *supra*). Spores for each strain are harvested into water with 0.01% Tween 20 to a concentration of  $5 \times 10^4$  spores/ml and the culture is divided. *Id.* The test compound is added to one culture to a final concentration of 20-100 µg/ml. Solvent only is added to  
25 the second culture. Rice infection assays are performed using Indica rice cultivar CO39 essentially as described in Valent *et al.*, *supra*. Two-week-old seedlings of cultivar CO39 are sprayed with 12 ml of conidial suspension. The inoculated plants are incubated in a dew chamber at 27°C in the dark for 36 hours, and transferred to a growth chamber (27 °C 12 hours/21 °C 12 hours 70% humidity) for an additional 5.5 days. Leaf samples  
30 are examined at 5 days post-inoculation to determine the extent of pathogenicity of the mutant and wild-type fungal strains as compared to their untreated control samples.

Alternatively, antipathogenic activity can be assessed using an excised leaf pathogenicity assay. Spore suspensions are prepared in water only to a concentration of  $5 \times 10^4$  spores/ml and the culture is divided. The test compound is added to one culture to a final concentration of 20-100 µg/ml. Solvent only is added to the second culture.

5 Detached leaf assays are performed by excising 1 cm segments of rice leaves from Indica rice cultivar CO39 and placing them on 1% agarose in water. 10 µl of each spore suspension is placed on the leaf segments and the samples are incubated at 25°C for 5 days in the dark. Leaf samples are examined at 5 days post-inoculation to determine the extent of pathogenicity of the mutant and wild-type fungal strains as compared to their

10 untreated control samples.

### Example 13

#### In Vivo Cell Based Assay Screening Protocol with a Fungal Strain Containing a Mutant Form of Ornithine Carbamoyltransferase with Reduced Activity

15 The effect of test compounds on the growth of wild-type fungal cells and mutant fungal cells having a mutant OCTase gene is measured and compared as follows. *Magnaporthe grisea* fungal cells containing a mutant form of the OCTase gene resulting in reduced activity, such as a transposon insertion mutation in a regulatory region of the gene or a promoter truncation mutation that reduces expression, are grown under standard

20 fungal growth conditions that are well known and described in the art. A promoter truncation is made by deleting a portion of the promoter upstream of the transcription start site using standard molecular biology techniques that are well known and described in the art (Sambrook *et al.*, *supra*).

The mutant and wild-type *Magnaporthe grisea* spores are harvested from cultures 25 grown on complete agar medium containing L-arginine (Sigma) after growth for 10-13 days in the light at 25°C using a moistened cotton swab. The concentration of spores is determined using a hemacytometer and spore suspensions are prepared in a minimal growth medium to a concentration of  $2 \times 10^5$  spores per ml. Approximately  $4 \times 10^4$  spores are added to each well of 96-well plates to which a test compound is added (at varying 30 concentrations). The total volume in each well is 200µl. Wells with no test compound present (growth control), and wells without cells are included as controls (negative

control). The plates are incubated at 25°C for seven days and optical density measurements at 590nm are taken daily. Wild-type cells are screened under the same conditions.

The effect of each test compound on the mutant and wild-type fungal strains is measured against the growth control and the percent of inhibition is calculated as the OD<sub>590</sub> (fungal strain plus test compound)/OD<sub>590</sub> (growth control) x 100. The percent growth inhibition as a result of each of the test compounds on the mutant and wild-type cells is compared. Compounds that show differential growth inhibition between the mutant and the wild-type cells are identified as potential antifungal compounds. Similar protocols may be found in Kirsch & DiDomenico, *supra*

Test compounds that produce a differential growth response between the mutant and wild-type fungal strains are further tested for antipathogenic activity. Each *M. grisea* strain is grown as described for spore production on oatmeal agar media (Talbot *et al.*, *supra*). Spores for each strain are harvested into water with 0.01% Tween 20 to a concentration of 5x10<sup>4</sup> spores/ml and the culture is divided. *Id.* The test compound is added to one culture to a final concentration of 20-100 µg/ml. Solvent only is added to the second culture. Rice infection assays are performed using Indica rice cultivar CO39 essentially as described in Valent *et al.*, *supra*. Two-week-old seedlings of cultivar CO39 are sprayed with 12 ml of conidial suspension. The inoculated plants are incubated in a dew chamber at 27°C in the dark for 36 hours, and transferred to a growth chamber (27 °C 12 hours/21 °C 12 hours at 70% humidity) for an additional 5.5 days. Leaf samples are examined at 5 days post-inoculation to determine the extent of pathogenicity of the mutant and wild-type fungal strains as compared to their untreated control samples.

Alternatively, antipathogenic activity can be assessed using an excised leaf pathogenicity assay. Spore suspensions are prepared in water only to a concentration of 5x10<sup>4</sup> spores/ml and the culture is divided. The test compound is added to one culture to a final concentration of 20-100 µg/ml. Solvent only is added to the second culture. Detached leaf assays are performed by excising 1 cm segments of rice leaves from Indica rice cultivar CO39 and placing them on 1% agarose in water. 10 µl of each spore suspension is place on the leaf segments and the samples are incubated at 25°C for 5 days in the dark. Leaf samples are examined at 5 days post-inoculation to determine the

extent of pathogenicity of the mutant and wild-type fungal strains as compared to their untreated control samples.

#### Example 14

5      In Vivo Cell Based Assay Screening Protocol with a Fungal Strain Containing a Mutant  
Form of an Arginine Biosynthetic Gene that Lacks Activity

The effect of test compounds on the growth of wild-type fungal cells and mutant fungal cells having a mutant form of a gene in the arginine biosynthetic pathway is measured and compared as follows. *Magnaporthe grisea* fungal cells containing a  
10     mutant form of a gene that lacks activity in the arginine biosynthetic pathway (e.g. argininosuccinate lyase having a transposon insertion) are grown under standard fungal growth conditions that are well known and described in the art. *Magnaporthe grisea* spores are harvested from cultures grown on complete agar medium containing L-arginine (Sigma) after growth for 10-13 days in the light at 25°C using a moistened  
15     cotton swab. The concentration of spores is determined using a hemacytometer and spore suspensions are prepared in a minimal growth medium containing L-arginine to a concentration of  $2 \times 10^5$  spores per ml.

Approximately  $4 \times 10^4$  spores or cells are harvested and added to each well of 96-well plates to which growth media is added in addition to an amount of test compound (at  
20     varying concentrations). The total volume in each well is 200µl. Wells with no test compound present, and wells without cells are included as controls. The plates are incubated at 25°C for seven days and optical density measurements at 590nm are taken daily. Wild-type cells are screened under the same conditions.

The effect of each compound on the mutant and wild-type fungal strains is measured against the growth control and the percent of inhibition is calculated as the  
25      $OD_{590}$  (fungal strain plus test compound) /  $OD_{590}$  (growth control) x 100. The percent of growth inhibition as a result of each of the test compounds on the mutant and the wild-type cells are compared. Compounds that show differential growth inhibition between the mutant and the wild-type cells are identified as potential antifungal compounds.  
30     Similar protocols may be found in Kirsch & DiDomenico, *supra*.

Test compounds that produce a differential growth response between the mutant and wild-type fungal strains are further tested for antipathogenic activity. Each *M. grisea* strain is grown as described for spore production on oatmeal agar media (Talbot *et al.*, *supra*). Spores for each strain are harvested into water with 0.01% Tween 20 to a 5 concentration of  $5 \times 10^4$  spores/ml and the culture is divided. *Id.* The test compound is added to one culture to a final concentration of 20-100 µg/ml. Solvent only is added to the second culture. Rice infection assays are performed using Indica rice cultivar CO39 essentially as described in Valent *et al.*, *supra*. Two-week-old seedlings of cultivar CO39 are sprayed with 12 ml of conidial suspension. The inoculated plants are incubated in a 10 dew chamber at 27°C in the dark for 36 hours, and transferred to a growth chamber (27° C 12 hours/21° C 12 hours at 70% humidity) for an additional 5.5 days. Leaf samples are examined at 5 days post-inoculation to determine the extent of pathogenicity of the mutant and wild-type fungal strains as compared to their untreated control samples.

Alternatively, antipathogenic activity can be assessed using an excised leaf 15 pathogenicity assay. Spore suspensions are prepared in water only to a concentration of  $5 \times 10^4$  spores/ml and the culture is divided. The test compound is added to one culture to a final concentration of 20-100 µg/ml. Solvent only is added to the second culture. Detached leaf assays are performed by excising 1 cm segments of rice leaves from Indica 20 rice cultivar CO39 and placing them on 1% agarose in water. 10 µl of each spore suspension is placed on the leaf segments and the samples are incubated at 25°C for 5 days in the dark. Leaf samples are examined at 5 days post-inoculation to determine the extent of pathogenicity of the mutant and wild-type fungal strains as compared to their untreated control samples.

25

### Example 15

#### In Vivo Cell Based Assay Screening Protocol with a Fungal Strain Containing a Mutant Form of an Arginine Biosynthetic Gene with Reduced Activity

The effect of test compounds on the growth of wild-type fungal cells and mutant 30 fungal cells having a mutant form of a gene in the arginine biosynthetic pathway is measured and compared as follows. *Magnaporthe grisea* fungal cells containing a mutant form of a gene resulting in reduced protein activity in the arginine biosynthetic

pathway (e.g. argininosuccinate lyase having a promoter truncation that reduces expression), are grown under standard fungal growth conditions that are well known and described in the art. Mutant and wild-type *Magnaporthe grisea* spores are harvested from cultures grown on complete agar medium containing L-arginine (Sigma) after 5 growth for 10-13 days in the light at 25°C using a moistened cotton swab. The concentration of spores is determined using a hemacytometer and spore suspensions are prepared in a minimal growth medium to a concentration of  $2 \times 10^5$  spores per ml.

Approximately  $4 \times 10^4$  spores or cells are harvested and added to each well of 96-well plates to which growth media is added in addition to an amount of test compound (at 10 varying concentrations). The total volume in each well is 200µl. Wells with no test compound present, and wells without cells are included as controls. The plates are incubated at 25°C for seven days and optical density measurements at 590 nm are taken daily. Wild-type cells are screened under the same conditions. The effect of each compound on the mutant and wild-type fungal strains is measured against the growth 15 control and the percent of inhibition is calculated as the  $OD_{590}$  (fungal strain plus test compound) /  $OD_{590}$  (growth control) x 100. The percent of growth inhibition as a result of each of the test compounds on the mutant and wild-type cells are compared. Compounds that show differential growth inhibition between the mutant and the wild-type cells are identified as potential antifungal compounds. Similar protocols may be 20 found in Kirsch & DiDomenico, *supra*.

Test compounds that produce a differential growth response between the mutant and wild-type fungal strains are further tested for antipathogenic activity. Each *M. grisea* strain is grown as described for spore production on oatmeal agar media (Talbot *et al.*, *supra*). Spores for each strain are harvested into water with 0.01% Tween 20 to a 25 concentration of  $5 \times 10^4$  spores/ml and the culture is divided. *Id.* The test compound is added to one culture to a final concentration of 20-100 µg/ml. Solvent only is added to the second culture. Rice infection assays are performed using Indica rice cultivar CO39 essentially as described in Valent *et al.*, *supra*. Two-week-old seedlings of cultivar CO39 are sprayed with 12 ml of conidial suspension. The inoculated plants are incubated in a 30 dew chamber at 27°C in the dark for 36 hours, and transferred to a growth chamber (27° C 12 hours/21° C 12 hours at 70% humidity) for an additional 5.5 days. Leaf samples are

examined at 5 days post-inoculation to determine the extent of pathogenicity of the mutant and wild-type fungal strains as compared to their untreated control samples.

Alternatively, antipathogenic activity can be assessed using an excised leaf pathogenicity assay. Spore suspensions are prepared in water only to a concentration of 5  $5 \times 10^4$  spores/ml and the culture is divided. The test compound is added to one culture to a final concentration of 20-100 µg/ml. Solvent only is added to the second culture. Detached leaf assays are performed by excising 1 cm segments of rice leaves from Indica rice cultivar CO39 and placing them on 1% agarose in water. 10 µl of each spore suspension is placed on the leaf segments and the samples are incubated at 25°C for 5 days in the dark. Leaf samples are examined at 5 days post-inoculation to determine the extent of pathogenicity of the mutant and wild-type fungal strains as compared to their untreated control samples.

#### Example 16

15        In Vivo Cell Based Assay Screening Protocol with a Fungal Strain Containing a Heterologous OCTase Gene.

The effect of test compounds on the growth of wild-type fungal cells and fungal cells lacking a functional endogenous OCTase gene and containing a heterologous OCTase gene is measured and compared as follows. Wild-type *M. grisea* fungal cells and *M. grisea* fungal cells lacking an endogenous OCTase gene and containing a heterologous OCTase gene from *Neurospora crassa* (Genbank Accession No. CAB91256), are grown under standard fungal growth conditions that are well known and described in the art.

An *M. grisea* strain carrying a heterologous OCTase gene is made as follows. An 25 *M. grisea* strain is made with a nonfunctional endogenous OCTase gene, such as one containing a transposon insertion in the native gene that abolishes protein activity. A construct containing a heterologous OCTase gene is made by cloning a heterologous OCTase gene, such as from *Neurospora crassa*, into a fungal expression vector containing a *trpC* promoter and terminator (e.g. Carroll *et al.*, 41 *Fungal Gen. News Lett.* 30 22 (1994) (describing pCB1003) using standard molecular biology techniques that are well known and described in the art (Sambrook *et al.*, *supra*). The vector construct is

used to transform the *M. grisea* strain lacking a functional endogenous OCTase gene. Fungal transformants containing a functional OCTase gene are selected on minimal agar medium lacking L-arginine, as only transformants carrying a functional OCTase gene grow in the absence of L-arginine.

5 Wild-type strains of *M. grisea* and strains containing a heterologous form of OCTase are grown under standard fungal growth conditions that are well known and described in the art. *M. grisea* spores are harvested from cultures grown on complete agar medium after growth for 10 - 13 days in the light at 25° C using a moistened cotton swab. The concentration of spores is determined using a hemacytometer and spore  
10 suspensions are prepared in a minimal growth medium to a concentration of  $2 \times 10^5$  spores per ml.

15 Approximately  $4 \times 10^4$  spores or cells are harvested and added to each well of 96-well plates to which growth media is added in addition to an amount of test compound (at varying concentrations). The total volume in each well is 200 µl. Wells with no test  
compound present, and wells without cells are included as controls. The plates are  
incubated at 25° C for seven days and optical density measurements at 590 nm are taken daily. The effect of each compound on the wild-type and heterologous fungal strains is measured against the growth control and the percent of inhibition is calculated as the  
OD<sub>590</sub> (fungal strain plus test compound) / OD<sub>590</sub> (growth control) x 100. The percent of  
20 growth inhibition as a result of each of the test compounds on the wild-type and heterologous fungal strains are compared. Compounds that show differential growth inhibition between the wild-type and heterologous strains are identified as potential antifungal compounds with specificity to the native or heterologous OCTase gene products. Similar protocols may be found in Kirsch & DiDomenico, *supra*.

25 Test compounds that produce a differential growth response between the strain containing a heterologous gene and strain containing a fungal gene are further tested for antipathogenic activity. Each *M. grisea* strain is grown as described for spore production on oatmeal agar media (Talbot *et al.*, *supra*). Spores for each strain are harvested into water with 0.01% Tween 20 to a concentration of  $5 \times 10^4$  spores/ml and the culture is  
30 divided. *Id.* The test compound is added to one culture to a final concentration of 20-100 µg/ml. Solvent only is added to the second culture. Rice infection assays are performed

using Indica rice cultivar CO39 essentially as described in Valent *et al.*, *supra*. Two-week-old seedlings of cultivar CO39 are sprayed with 12 ml of conidial suspension. The inoculated plants are incubated in a dew chamber at 27°C in the dark for 36 hours, and transferred to a growth chamber (27 °C 12 hours/21 °C 12 hours at 70% humidity) for an additional 5.5 days. Leaf samples are examined at 5 days post-inoculation to determine the extent of pathogenicity of the wild-type and heterologous fungal strains as compared to their untreated control samples.

Alternatively, antipathogenic activity can be assessed using an excised leaf pathogenicity assay. Spore suspensions are prepared in water only to a concentration of  $5 \times 10^4$  spores/ml and the culture is divided. The test compound is added to one culture to a final concentration of 20-100 µg/ml. Solvent only is added to the second culture. Detached leaf assays are performed by excising 1 cm segments of rice leaves from Indica rice cultivar CO39 and placing them on 1% agarose in water. 10 µl of each spore suspension is placed on the leaf segments and the samples are incubated at 25°C for 5 days in the dark. Leaf samples are examined at 5 days post-inoculation to determine the extent of pathogenicity of the wild-type and heterologous fungal strains as compared to their control samples.

#### Example 17

##### Pathway Specific *In Vivo* Assay Screening Protocol

Compounds are tested as candidate antibiotics as follows. *Magnaporthe grisea* fungal cells are grown under standard fungal growth conditions that are well known and described in the art. Wild-type *M. grisea* spores are harvested from cultures grown on oatmeal agar media after growth for 10-13 days in the light at 25°C using a moistened cotton swab. The concentration of spores is determined using a hemocytometer and spore suspensions are prepared in a minimal growth medium and a minimal growth medium containing L-arginine (Sigma) to a concentration of  $2 \times 10^5$  spores per ml. The minimal growth media contains carbon, nitrogen, phosphate, and sulfate sources, and magnesium, calcium, and trace elements (for example, see innoculating fluid in Example 7). Spore suspensions are added to each well of a 96-well microtiter plate (approximately  $4 \times 10^4$  spores/well). For each well containing a spore suspension in minimal media, an

additional well is present containing a spore suspension in minimal medium containing L-arginine.

Test compounds are added to wells containing spores in minimal media and minimal media containing L-arginine. The total volume in each well is 200 $\mu$ l. Both 5 minimal media and L-arginine containing media wells with no test compound are provided as controls. The plates are incubated at 25°C for seven days and optical density measurements at 590nm are taken daily. A compound is identified as a candidate for an antibiotic acting against the L-arginine biosynthetic pathway when the observed growth in the well containing minimal media is less than the observed growth in the well 10 containing L-arginine as a result of the addition of the test compound. Similar protocols may be found in Kirsch & DiDomenico, *supra*.

Published references and patent publications cited herein are incorporated by reference as if terms incorporating the same were provided upon each occurrence of the individual reference or patent document. While the foregoing describes certain 15 embodiments of the invention, it will be understood by those skilled in the art that variations and modifications may be made that will fall within the scope of the invention. The foregoing examples are intended to exemplify various specific embodiments of the invention and do not limit its scope in any manner.